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THE EFFECT OF A CALCIUM ANTAGONIST (D600) ON ISOPRENALINE-INDUCED MYOCARDIAL NECROSIS IN THE RAT

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Summary.—The effect of inhibiting isoprenaline-induced intracellular calcium accumulation on the degree of damage produced in the rat myocardium by this amine has been investigated by simultaneously dosing rats with the calcium antagonistic drug D600. The degree of myocardial necrosis produced in animals given isoprenaline alone and those given D600 was measured by the use of a standard point counting method to show absence of formazan from dead muscle fibres in sections treated to demonstrate succinic dehydrogenase. The use of the calcium antagonistic drug D600 considerably reduced the degree of myocardial damage produced by a standard dose of isoprenaline bitartrate. This was associated with a decrease in the isoprenaline-induced cellular calcium uptake. The results are discussed in relation to the possible protection conferred by lowering of the calcium influx at the cell membrane and the maintenance of the cells' high-energy phosphates at a level which permits "normal" cell function.

CONVINCING EVIDENCE exists that myocardial ischaemia is associated with a release of endogenous catecholamine in the myocardium and a concomitant increase in plasma and urine catecholamine levels (Ceremuzynski, Staszewska-Barczak and Herbaczynska-Cedro, 1969; Gazes, Richardson and Woods, 1959). Such local and systemic increases in catecholamine levels could affect the performance and survival of myocardial fibres in a variety of ways (Fitzgerald, 1972). In this communication attention is focused on only one of these: the accumulation of intracellular calcium in heart muscle and its possible deleterious effects on the myocardium.

Calcium ions are considered to play a central role in the excitation–contraction coupling process of the heart (Katz, 1970; Langer, 1968; Reuter, 1974), and both calcium deficiency and calcium overload in the myocardial cell have recently been implicated in the development of contractile failure (Dhalla, 1976). Thus disturbances in calcium movements within the cardiac cell could play an important role in the pathogenesis of heart failure.

Through their inotropic action catecholamines have been shown to be involved in the elevation of intracellular calcium in the heart (Bloom and Davis, 1972; Reuter, 1974). Fleckenstein *et al.* (1974) have shown that high levels of catecholamine produced an increased availability of intracellular calcium which was suggested to be associated with myocardial damage. We have been interested in the possibility that excess levels of intracellular calcium might be causally associated with the degree of structural damage to the myocardium.

The model we have used to examine this question is the well-known one of isoprenaline-induced necrosis in the rat heart (Chappel *et al.*, 1959). Although the isoprenaline model cannot be regarded as the homologue of events leading to myocardial infarction in man or experimental animals, it nevertheless has some attractions. Firstly, since isoprenaline is a sympathomimetic amine, it may well operate within

the heart much as endogenous catecholamines do. Secondly, the morphological changes which it produces in the rat heart, those of subendocardial necrosis, resemble the changes associated with laminar infarction in man, and this suggests the pathogenesis of the 2 lesions may be similar. Collins and Billings (1976) have shown that isoprenaline in a single dose induces impairment in perfusion of the subendocardial myocardium and that this defect in perfusion corresponds in distribution to the myocardial necrosis produced by a similar dose of isoprenaline, and Hoffman and Buckberg (1975) have shown that reduction in subendocardial perfusion in the human heart can take place with anatomically normal epicardial vessels.

We have shown previously (Woolf *et al.*, 1976) that isoprenaline-induced myocardial necrosis in the rat can be quantitated objectively on the basis of a simple enzyme histochemical technique. This paper records our experience of the effect of inhibiting the isoprenaline-induced increase in myocardial calcium uptake by the simultaneous administration of the calcium antagonist drug D600.

MATERIALS AND METHODS

Eighty male albino rats weighing approximately 250 g were used in this study. All were of the Charles River CD strain (Sprague–Dawley-derived).

The amine used was isoprenaline bitartrate (a 99% pure laevo-isomer manufactured by Aldrich Chemical Co. Inc.). The amine was dissolved in distilled water and was given by s.c. injection into the right flank. The solution used contained 10 mg/ml and a standard dose of 20 mg/kg body wt was given.

In half the animals the isoprenaline was given simultaneously with D600. The D600 (manufactured by Knoll AG. Chemische Fabriken, W. Germany) was given by s.c. injection into the left flank at a dose of 10 mg/kg body wt.

The 80 rats were killed 72 h after treatment by exposing them to carbon dioxide. The hearts were removed while still beating and immediately arrested in 0·15M KCl at 4°. A block taken through the apices of both ventricles was then snap-frozen in isopentane at −70°. Frozen sections were prepared from these blocks as described previously (Woolf *et al.*, 1976). These were then treated to demonstrate succinate dehydrogenase using nitro-blue tetrazolium as the hydrogen acceptor (Chayen *et al.*, 1969). The specificity of this method was shown by the complete inhibition of formazan production in sections incubated in a medium to which malonate had been added. Necrotic muscle fibres showed no evidence of formazan deposition, thus allowing easy distinction between viable and necrotic muscle.

The degree of damage in sections of myocardium was determined by the use of a standard point counting method carried out with a Zeiss 25-point graticule. The ratio of points falling in necrotic fibres with no demonstrable enzyme content to those falling on viable fibres as judged by their normal enzyme content was calculated using the whole section, which in practice meant a minimum of 1800 points.

RESULTS

In the animals dosed with both D600 and isoprenaline the percentage loss of heart muscle fibres ranged from 0 to 17% with a mean of 3%. In those where isoprenaline alone was given the percentage ranged from 5 to 48% with a mean of 22%. Statistical analysis shows the difference in the raw data to be significant ($P<0·001$; Fig. 1).

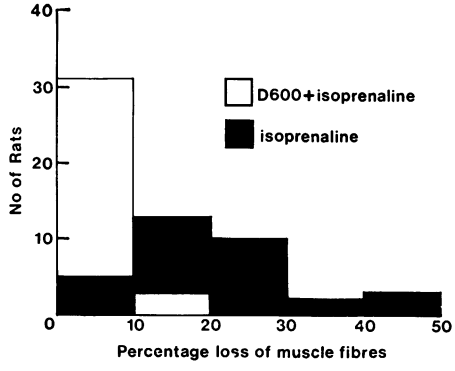

FIG. 1.—Effect of dosing with D600.

DISCUSSION

These results show that administering D600 simultaneously with isoprenaline markedly inhibits the damage produced in the myocardium by a standard dose of the amine. D600 produced a considerably

higher degree of inhibition that that previously found with the antilipolytic agent 5-fluoro-nicotinic acid (Woolf *et al.*, 1977) and in our experience was equal to that found using adrenergic-blocking agents.

The problem of interpretation arises from the fact that we still do not understand completely the mechanisms of isoprenaline-induced myocardial necrosis despite much work within this field. A number of possible pathogenetic mechanisms have been implicated. These include relative coronary insufficiency due to decreased diastolic compliance (Rona *et al.*, 1959; Buckberg and Gordon, 1973; Collins and Billings, 1976); metabolic impairment due to the presence of unfavourable substrates, *e.g.* excess free fatty acids (Opie, 1970; Kjekshus and Mjøs, 1972; Woolf *et al.*, 1977). Since the protective agent D600 used in this study is a calcium-flux inhibitor, the effects of isoprenaline on the cellular control of calcium must obviously be of primary importance in one's consideration of this model system.

Isoprenaline is known to mediate its inotropic action by induction of the adenyl-cyclase-cyclic AMP system (Robison, Butcher and Sutherland, 1971; Lefkowitz *et al.*, 1976), which in turn augments the transmembrane influx of calcium during the upstroke phase of the cardiac action potential (Reuter, 1974). Calcium ions quantitatively control excitation–contraction coupling by regulating the amount of adenosine triphosphate (ATP) which is split by the calcium-dependent myofibrillar ATPase. Many data exist which suggest that the isoprenaline-induced calcium influx leads to overstimulation of this process, the final result being the breakdown of cellular calcium control with the concomitant loss of high energy phosphates.

Fleckenstein (1971) has found that a single dose of isoprenaline (30 mg/kg) administered to rats induces ^{45}Ca uptake into the ventricular muscle of the heart. A second similar experiment by the same workers has shown the ^{45}Ca uptake to be associated with a 50% loss of ATP and 85% loss of creatine phosphate (CP); all these hearts developed severe myocardial necrosis.

Electron microscope studies provide evidence that isoprenaline produces structural changes in both the myofibrils and the mitochondria of the cardiac cell. Bloom and Cancilla (1969) and Csapo, Dŭsek and Rona (1972) have found that hypercontraction of the myofilaments occurs within 6 min of a single injection of isoprenaline in rats. Electron-dense deposits, believed to be aggregates of calcium, appeared in the mitochondria within 15 min. This sequence of morphological events suggests early damage to the sarcoplasmic reticulum with release of calcium, and uptake of this calcium by metabolically active mitochondria.

Otto and Ontko (1978) have shown in liver cells that calcium accumulation by mitochondria appears to be a prerequisite for the enhanced uptake and oxidation of free fatty acids, and Jennings and Ganote (1972) have found that the amorphous matrix densities in the mitochondria of irreversibly damaged heart cells contain a high proportion of lipid in addition to calcium. Both calcium accumulation and lipid uptake are energy-dependent processes and take place at the expense of ATP production (Lehninger, Carofoli and Rossi, 1967). This decrease in mitochondrial ATP production could result in the cells' failure to maintain adequate high-energy phosphates for "normal" function and perhaps suggests a relationship between cellular free fatty acids and calcium accumulation.

As mentioned previously, D600 is a calcium flux antagonist drug (Fleckenstein *et al.*, 1969) and has been shown to lower the cell-membrane conductance for calcium ions, possibly by inhibiting the low-affinity binding sites for calcium which are located on the external surface of the membrane and are related to calcium entry (Williamson, Scarpa and Woodrow, 1973). Therefore D600 could reverse the inotropic effect of isoprenaline which is responsible for augmenting contraction

simply by decreasing the availability of calcium ions at the plasma membrane.

Verapamil, of which D600 is the methoxy derivative, has been found by Nayler, Grau and Slade (1976) to protect isolated rabbit hearts rendered hypoxic by anaerobic substrate-free perfusion. Verapamil reduced the amount of ultrastructural damage and decreased the rate at which the hypoxic muscle was depleted of its endogenous stores of ATP and CP. However, it did not prevent the hypoxic muscle from gaining calcium. Verapamil is known to affect the slow-moving calcium channel; *i.e.* it directly affects the physiological control of calcium movement. This therefore suggests (within the above-mentioned experimental conditions) that there is a major alteration in membrane permeability towards calcium, which itself is possibly due to a lesion in the membrane.

Using an *in-vivo* system, we have shown D600 to be effective in preventing isoprenaline-induced myocardial necrosis, which suggests that this lesion is indeed caused by calcium accumulation and involves the slow membrane channel for calcium. The accumulation of calcium alone, without any major alteration in membrane permeability, might well affect energy-producing mechanisms within the cells and this in itself could lead to plasma membrane damage which in turn could still further increase the membrane permeability to calcium. Isoprenaline does not appear to damage the plasma membrane directly but may do so indirectly through the interrelated effects of calcium accumulation and ATP depletion, the final result being cellular necrosis.

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